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# Developmental regulation of the plant mitochondrial matrix located HSP70 chaperone and its role in protein import

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**Abstract Changes in the level of the mitochondrial chaperone mtHSP70 have been investigated in pea (*Pisum sativum*) leaf mitochondria by Western blot analysis and quantified by scanning densitometry. As pea leaves develop (from 6 days to 30 days of age) the levels of mtHSP70 decrease. Analysis of the levels of the α subunit of the $F_1$ATPase show that the levels of this protein remain constant throughout the same developmental period, whereas the levels of the alternative oxidase increase. In vitro import of the alternative oxidase precursor protein into pea leaf mitochondria from day 6 to day 30 leaves and quantification by scanning densitometry indicates that protein import efficiency decreases with increasing maturity of the plant cell. Results are discussed in terms of how changing levels of the mtHSP70 chaperone, as a result of plant cell development, influence the efficiency of protein import.**



*Key words:* Mitochondria; Import; HSP; *Pisum sativum*; Chaperone; Development

## 1. Introduction

In eukaryotic cells, the role of mitochondria is to generate both ATP (through the process of oxidative phosphorylation) and carbon skeletons (via the Krebs cycle) for biosynthetic purposes. A large majority (90–95%) of mitochondrial proteins are encoded by the nucleus of the cell, synthesised on cytosolic ribosomes and subsequently imported into the mitochondria [1]. Although rapid advances have been made recently in our understanding of the mechanisms by which proteins can be imported into *Saccharomyces cerevisiae* (yeast) and *Neurospora* mitochondria (reviewed in [1]), in comparison with these systems, relatively little is known about the precise components involved in protein import in higher plant mitochondria [2]. Recently, a component of the plant mitochondrial outer membrane import apparatus has been identified on the basis of immunological cross-reactivity between *Neurospora*, *S. cerevisiae* and plant outer membrane proteins [3]. This work indicated that components of protein import may have been conserved through evolution. One of the final steps in the import process is the engagement of the protein being imported with a molecular chaperone – a heat shock protein of 70 kDa (mtHSP70). This step was first observed in *S. cerevisiae* [4] and subsequently found to be essential for the translocation and re-folding of imported proteins in this organism [5]. The exact mechanism by which mtHSP70 is able to drive protein import is unclear. There are two potential mechanisms at present being discussed, these being the 'Brownian ratchet' and a 'translocation motor' model [6,7]. The current evidence (recently reviewed in [8]) suggests that the former mechanism could account for the initial insertion and capture of the mitochondrial presequence whilst the translocation motor could explain the process by which the precursor protein is actually taken into the mitochondrial matrix.

Mitochondrial HSP70 genes have been identified from a number of other plant species including pea leaf [9], bean [10] and potato and tomato [11]. Sequence comparison has revealed that these plant HSP70s have high sequence similarity between themselves and also with the mitochondrially located HSP70s from *S. cerevisiae* [12] and *Schizosaccharomyces pombe* [13]. In both of these organisms the mtHSP70 chaperone plays a central and critical role in cell viability, indeed cell death occurs in *S. cerevisiae* mutants lacking this gene [5].

By analogy with the yeast mtHSP70 the homologous plant gene products have been proposed to have a role in protein import. Indirect evidence to support this comes from co-import experiments of proteins into pea leaf tissue [14]. In these type of experiments, in vitro import of proteins into plant mitochondria was enhanced if mtHSP70 is added to the import reaction. Previous work from our laboratory has shown that the activities of several mitochondrially located enzymes alter during the development of the leaf [15]. In addition, we have proposed that the levels of mtHSP70 may be limiting mitochondrial import in mature plant tissue.

The results presented here demonstrate that the levels of the plant mtHSP70 decline as the plant ages. The decline in mtHSP70 levels was correlated with a reduction in the efficiency of mitochondrial protein import. In addition, we undertook a study of the correlation between the relative efficiency of the import process and levels of the mtHSP70 protein.

## 2. Materials and methods

### 2.1. Plant material

*Pisum sativum* L. cultivar Feltham First (Sharpes Seeds, Sleaford) seeds were bleached in 0.1% sodium hypochlorite for 10 min, rinsed under running water and then aerated overnight. The imbibed seeds were sown in SHL potting compost (William Sinclair, Lincoln) and grown for 6–30 days under a 12 h light/dark cycle with a light intensity of 700 μmol $m^{-2}$ $s^{-1}$. Plants were watered once a day with tap water.

### 2.2. Isolation of mitochondria

Mitochondria were isolated by differential centrifugation from the leaf tissue of *P. sativum* and purified using a 23% (v/v) Percoll gradient containing a linear 0–10% (w/v) gradient of PVP-40 as previously described [15]. This method allows the isolation of mitochondria free from other contaminating organelles such as chloroplasts and endoplasmic reticulum.

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*2.3. Resolution of proteins by SDS-PAGE*

The concentration of proteins was assayed using the BCA protein kit (Pierce and Warriner, Chester, UK). Equal amounts of protein (approximately 14 μg) were electrophoresed on 10% SDS-PAGE gels essentially according to Laemmli [16].

*2.4. Scanning densitometry*

SDS-PAGE gels and import autoradiographs were scanned on a Sharp JX-325 densitometer.

*2.5. Electroblotting to nitrocellulose membrane*

Electrophoresed proteins were transferred to nitrocellulose membrane (Schleicher and Schuell, 0.45 μm pore size) according to the method of Towbin et al. [17]. The transfer buffer contained 12.6 mM Tris buffer pH 7.5, 192 mM glycine and 10% methanol. The proteins were transferred at 100 V (constant voltage) for 1 h.

*2.6. Western blotting*

The filters were initially incubated with 3% (w/v) BSA and 2% (w/v) milk powder in phosphate buffered saline (PBS) (145 mM NaCl pH 7.2, 12.5 mM $Na_2HPO_4$, 2.5 mM $NaH_2PO_4$) in order to block unbound protein reactive sites. The filters were then washed in 0.6% (w/v) milk powder, 0.1% (v/v) Tween-20 buffer in PBS. The transferred proteins were probed with a series of primary antibodies. The three antibodies used were raised against the plant mtHSP70, the alternative oxidase or the α subunit of the $F_1$ATPase. Anti-PHSP1 was raised, in rabbit, against a synthetic peptide of 10 amino acids from the *P. sativum* mitochondrial HSP70 protein [9], which is located in the matrix or loosely associated with the inner membrane [18]. The antibodies against the alternative oxidase (AOA) were raised in mice [19]. The antibodies raised against the $F_1$ATPase protein was produced from mice that were injected with total mitochondrial protein [20]. The above antibodies were used at a dilution of 1 in 1000 in 3% (w/v) BSA and PBS buffer. The filters were washed as above, prior to incubation with the appropriate secondary antibodies, i.e. anti-mouse or anti-rabbit, linked to horseradish peroxidase enzyme, which were used at a concentration of 1 in 1000 diluted as for the primary antibodies. The filters were washed in blot rinse buffer (10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% (v/v) Tween-20) and the bound antibodies were detected using an enhanced chemiluminescence kit (Amersham International plc, Aylesbury, Bucks, UK).

*2.7. In vitro protein import*

The alternative oxidase cDNA [21] was cloned behind a T3 promoter and transcribed in vitro. The resulting mRNA was translated in the presence of [$^{35}$S]methionine (Amersham) in a nuclease-pretreated rabbit reticulocyte lysate (Promega). Import experiments were performed in a final volume of 220 μl in 0.3 M sorbitol, 5 mM $MgCl_2$, 12.5 mM HEPES (pH 7.4), 2 mM $KH_2PO_4$, 5 mM unlabelled methionine, 1 mM DTT, 1 mM $MnCl_2$, 1 mM ADP, 1 mM NADH, 2 mM ATP, 9 mM creatine phosphate and 120 μg/ml creatine kinase. 440 μg of purified mitochondrial protein was used per import reaction, which was performed in siliconised scintillation vials at 25°C for 30 min in a shaking water bath. Import was stopped by the addition of valinomycin to a final concentration of 2 μg/ml, and if required proteinase K was added to a final concentration of 20 μg/ml. Phenylmethylsulphonyl fluoride was then added to both proteinase K-treated and untreated samples to a final concentration of 1 mM. Mitochondria were re-isolated and resuspended in SDS sample buffer, and proteins were separated by SDS-PAGE gel electrophoresis on 10% gels as described above.

*2.8. Chemicals*

Unless stated otherwise all chemicals were purchased from Sigma (Poole, Dorset, UK) and were of the highest purity commercially available.

## 3. Results

*3.1. Developmental regulation of the mtHSP70 protein*

Mitochondria were isolated from pea leaves of 6–30 days of age and, following separation of proteins by SDS-PAGE, were probed by Western blot analysis with antibodies against the mtHSP70 (PHSP1), the α-$F_1$ATPase and the alternative oxidase. Fig. 1A shows that the levels of the mtHSP70 protein decrease as the tissue from which the mitochondria were isolated ages. The highest amounts were observed in mitochondria isolated from 6 day old leaf tissue (Fig. 1A, lane 1). Fig. 1D indicates that when the blots were quantified by scanning densitometry the levels of mtHSP70 decreased dramatically by 64% (from 7.2 to 2.5 units) during the period between 6 and 30 days. Fig. 1B,D reveals, however, that there was negligible variation in the levels of the α-$F_1$ATPase during the same period. The levels of this protein appeared to remain relatively constant throughout the developmental period studied. In contrast, when mitochondrial proteins were probed with antibodies against the alternative oxidase (Fig. 1C) the levels of the alternative oxidase protein increased with cell maturity up to day 30. This finding is in agreement with previously reported results from our laboratory [15] which showed that alternative oxidase activity increased during a similar developmental period. The results shown in Fig. 1B,C suggest strongly that the decreased levels of mtHSP70 observed as the leaf tissue increases in maturity (Fig. 1A) are an accurate

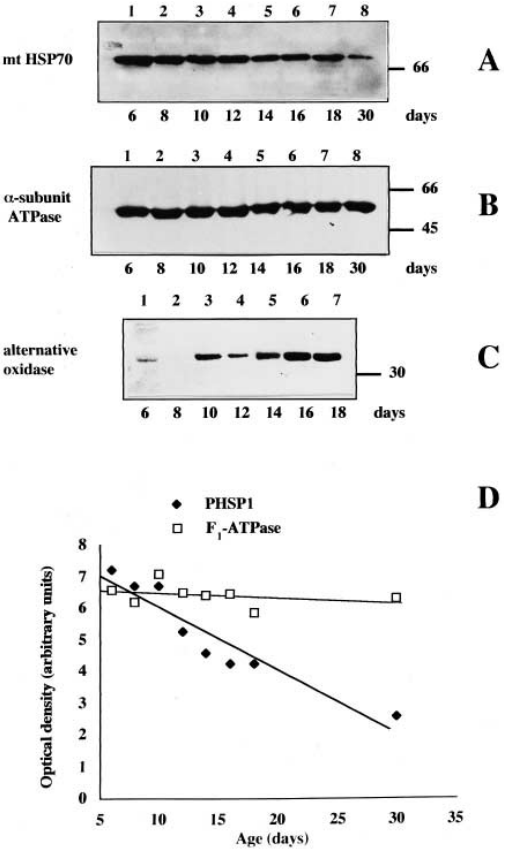


Fig. 1. The effects of pea leaf age upon the levels of mtHSP70, α subunit of the $F_1$ATPase and alternative oxidase proteins within isolated mitochondria. 14 μg of mitochondrial protein was loaded onto each lane of a 10% SDS-PAGE gel. The proteins were then transferred to nitrocellulose membrane and Western blot analysis was performed. Primary antibodies raised against the mtHSP70 (A), α subunit of the $F_1$ATPase (B) and the alternative oxidase (C) were used at a concentration of 1:1000 and were detected by peroxidase linked secondary antibodies at a dilution of 1:1000 followed by detection with the Amersham chemiluminescence kit. Molecular weights in kDa are indicated on the right hand side. The days indicate the age of the tissue after planting (from 6 to 30 days of age) from which mitochondria were isolated. Densitometry scans were performed upon the Western blots (D).

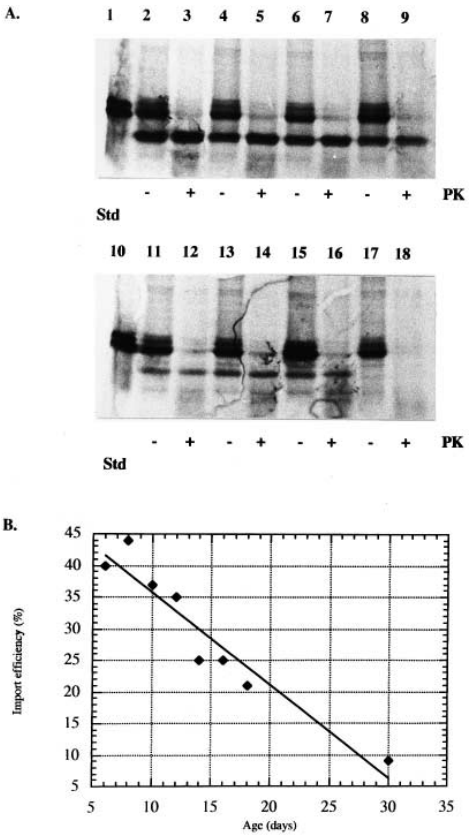


Fig. 2. In vitro import of the alternative oxidase into mitochondria isolated from pea leaf tissue of different ages and graphical representation of import efficiency. A: Lanes 1 and 10, alternative oxidase precursor standard alone. Mitochondria were isolated from pea leaf tissue that was 6 days of age (lanes 2, 3), 8 days (lanes 4, 5), 10 days (lanes 6, 7), 12 days (lanes 8, 9), 14 days (lanes 11, 12), 16 days (lanes 13, 14), 18 days (lanes 15, 16) and 30 days (lanes 17, 18). The alternative oxidase precursor was incubated with 220 μg of mitochondria in each case. In some cases proteinase K was added following import (designated +PK), in others proteinase K was absent (designated −PK). Precursor and mature refer to the unprocessed and processed forms of the alternative oxidase protein respectively. B: A graphical representation of the efficiency of protein import into the mitochondria isolated from pea leaves of different ages. Efficiency of import was determined as a measure of the ratio between the mature processed form of the protein versus the proteinase K-insensitive precursor form.

reflection of the changes occurring to this protein and are not merely a result of all proteins decreasing in amount as the tissue ages.

### 3.2. *Import of the alternative oxidase precursor protein into mitochondria isolated from pea leaf tissue of different ages*

To determine if the changes in the levels of mtHSP70 affect the efficiency of the import process, an investigation was performed into whether there was an alteration in the levels of protein import efficiency when the alternative oxidase precursor was imported into mitochondria isolated from pea leaf tissue of 6–30 days of age (Fig. 2A). Fig. 2A shows that the most efficient import of the alternative oxidase precursor was achieved when the protein was imported into mitochondria isolated from leaf tissue that was 6 days old whilst the least efficient import was seen in mitochondria that were isolated from tissue that was 30 days of age. Efficiency of import is a measure of the ratio between the mature processed form of the protein versus the proteinase K-insensitive precursor form. In those import reactions utilising mitochondria isolated from the youngest tissue (see Fig. 2, lanes 2 and 3) the efficiency of import is high whilst the import into more mature tissue (30 day old) (see Fig. 2, lanes 17 and 18) is less efficient. This result can be seen more clearly from the graphical representation of import efficiency versus age shown in Fig. 2B. There is an obvious decline in the efficiency of import into mitochondria that were isolated from older leaf tissue.

## 4. Discussion

In the present study, the developmental regulation of the mtHSP70 protein has been investigated. In a previous study of the developmental regulation of mitochondrial processes, we have demonstrated that mitochondria isolated from developing pea leaves undergo specific changes in respiratory activity as the tissue ages [15] indicating that such a system is suitable for use as a developmental tissue. Given the key role that mtHSP70 has been postulated to play in the control of plant mitochondrial import [22] we have also analysed the efficiency of protein import into pea leaf mitochondria isolated from increasing ages of tissue.

Interest in the possibility that mtHSP70 may be developmentally regulated arose from previous work in this laboratory which indicated that variations in the protein levels of the mtHSP70 (PHSP1) protein could have significant effects upon the efficiency of protein import [14]. Consequently levels of mtHSP70 could be considered to a major regulatory point within the import process. This was based upon a finding that increasing the intra-mitochondrial levels of mtHSP70 resulted in an increase in the import efficiency of a heterologous protein [14]. We have suggested that the low efficiency of import previously observed with plant mitochondria was not due to an inherent inefficiency of the plant protein import apparatus per se but could be attributed to the use, in import experiments, of mitochondria isolated from relatively mature tissue in which the endogenous levels of mtHSP70 were limiting. The high level of proteinase K-insensitive precursor protein observed in import reactions with such mitochondria was explained to be due to the arrest of the protein being imported after insertion of the presequence into the inner membrane [2]. Since the protein import process involves the transient association of the outer and inner membranes to form contact points [7,23] release of these contact points would result in the translocation of proteins into the intermembrane space, where it would be protected from the action of proteinase K [2].

The results in Fig. 1 strongly support the notion that mtHSP70 levels are considerably lower in mitochondria isolated from mature tissue when compared to younger tissue (a 64% decrease in mtHSP70 levels between day 6 and day 30). Since the levels of the α subunit of the $F_1$ATPase remained constant and the levels of the alternative oxidase increased during the same developmental period these results suggest that the decrease in mtHSP70 levels are real.

The observation that mtHSP70 levels decline during development can be correlated to similar changes in the activities of a number of respiratory proteins whose levels also change throughout plant development. During pea leaf development the activities of NADH dehydrogenase, succinate dehydrogenase and cytochrome *c* oxidase were found to remain fairly

constant from day 0 to day 18 of development [14]. Two other protein activities, namely the alternative oxidase and glycine decarboxylase, remained low between day 0 and day 6 but increased substantially as the tissue matured. We have suggested that the developmental regulation of the activities of these two proteins is in response to the changing role of the mitochondria as the tissue matures. Namely, that as the plant tissue matures, mitochondrial function switches from a more bioenergetic role to the provision of carbon skeletons [24–26]. The results presented in this paper demonstrate that as the role of mitochondria within the leaf tissue changes there is a corresponding reduction in the level of the mtHSP70 protein in addition to a reduction in the level of mitochondrial protein import efficiency. With respect to the role of the mtHSP70 protein during the import process, we have previously suggested [14] that there is a correlation between the levels of mtHSP70 and the efficiency of protein import. An enhancement in the efficiency of protein import (i.e. that there was more of the mature proteinase K-insensitive form of the heterologous probe present) was observed in mitochondria isolated from 14 day old pea leaves when mtHSP70 was co-imported with a heterologous probe. The results we have presented here extend this observation and show that the greatest protein import efficiency is seen with 6 day old mitochondria. In this experiment a higher proportion of the mature form of the protein was observed within 6 day old mitochondria than in the mitochondria isolated from older tissue. This finding compares favourably with the results presented in Fig. 1 which suggest that declining levels of mtHSP70 protein may be limiting the import process into plant mitochondria. We have recently generated transgenic tobacco plants that have greatly over-expressed levels of mtHSP70. It will be of interest to determine the import characteristics of the mitochondria isolated from transgenic tissue.

Whether there are other components of the import machinery which also decline during development and therefore reduce the levels of import remains to be investigated and will only become apparent once more components of the plant import apparatus are identified.

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